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(56) Documents Cited
Life Sci; Vol 62 (5), pp 389-396 (1998) Fukamauchi et al. Biochem Pharmacol; Vol 54(7), pp 833-839 (1997) Chang et al Mol Pharmacol; Vol 44(5), pp 940-949 (1993) Fukamauchi et al Febs Letts; Vol 276(1-2), pp 185-188 (1990) Wang et al.

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(54) Abstract Title
**Screening method**

(57) The method assesses the effectiveness of compounds for treatment of conditions characterised by a deficiency in number or function of membrane bound receptors and may be concerned with assessing putative agents for treatment of cognitive dysfunction conditions, including Alzheimer's disease. In the method, cells transfected with appropriate DNA are grown beyond the usual level to approach or reach equilibrium; the growth medium is then removed and the test agent added. A control is carried out using only the vehicle in which the test agent is dissolved. The samples are then incubated for, preferably, 72 hours, and an assay for the receptor or receptor type of interest is carried out. Comparison of results between control and test sample indicate the likely usefulness of the test compound. In a preferred embodiment, Chinese Hamster Ovary (CHO) cells are transfected with DNA for the muscarinic acetylcholine receptor, and receptor number is assayed using a [$^3$H]-NMS and [$^3$H]-QNB binding assay.



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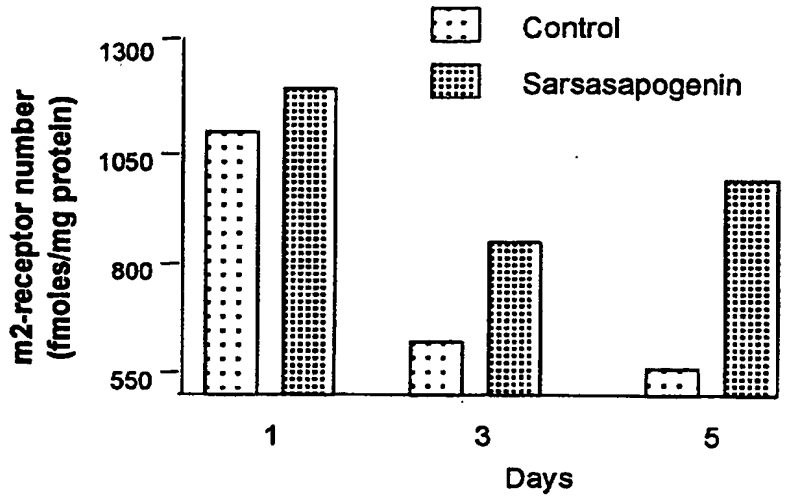


**Figure 1. The reduction in receptor number following prolonged incubation is prevented by incubation with sarsasapogenin (10μM)**

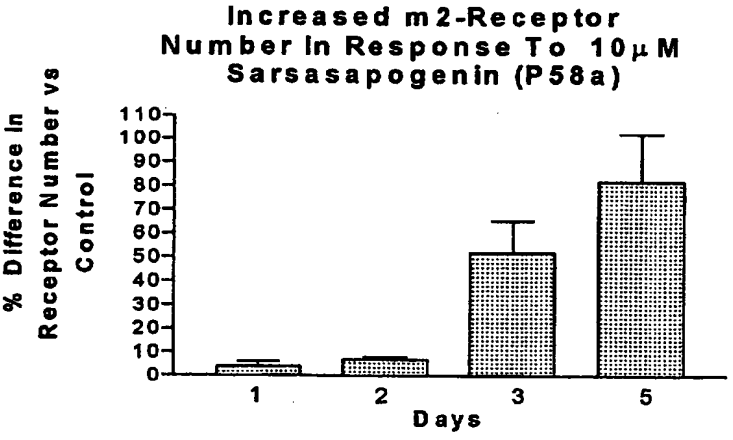


**Figure 2. The difference in number compared to control following prolonged incubation with sarsasapogenin (10μM)**

# SCREENING METHOD

This present invention relates to a predictive cell based assay *in vitro* to assess putative agents for the treatment of cognitive dysfunction and allied conditions including Alzheimer's disease.

This invention is also concerned with the assessment of agents for the treatment of conditions that are characterised by a deficiency in the number or function of membrane bound receptors.

In the following the present invention will be described principally with reference to muscarinic receptors and the treatment of Alzheimer's disease and senile dementia where deficiencies in the number of receptor types have been demonstrated.

However it is understood that the present invention relates generally to cells expressing other hormonal, neurotransmitter or autocrine or paracrine receptors for the treatment of conditions characterised by a deficiency in the number or function of membrane bound receptors.

Alzheimer's disease is one of the most common forms of dementia in old age, affecting approximately 11% of the population between 80 and 85 years of age and 26% of those older than 85. Whilst its aetiology remains unknown, the pathological manifestations of Alzheimer's disease are unmistakable: a reduction in cholinergic activity with characteristic senile (neuritic) plaques and neurofibrillary tangles in the limbic structures and associated neocortex in the brain.

Cholinergic pathways in the cerebral cortex and basal forebrain are compromised in patients with Alzheimer's disease and the cholinergic deficiency that results is associated with the cognitive deficits observed in these patients. Pharmacological enhancement of transmission in surviving cholinergic pathways therefore has been utilised as one method by which to improve cognitive function and relieve the symptoms of this condition. Various approaches have been investigated. The most widely studied has been the use of cholinesterase inhibitors. This largely symptomatic approach is aimed at enhancing cholinergic activity by inhibiting the breakdown of acetylcholine in the synaptic space between two neurones. Increasing

the concentration of the transmitter would be expected to improve transsynaptic transmission. Other approaches investigated include the use of muscarinic and nicotinic cholinoceptor agonists and neurotropic factors. The use of agonists on the face of it appears attractive, but should not be expected necessarily to enhance synaptic transmission. Indeed prolonged administration of agonists could result in receptor down regulation with the possibility of further impairment of synaptic transmission. The use of neurotropic factors is also an attractive strategy, but the ability of the ageing brain to significantly regenerate diseased or degenerating pathways is unknown.

Senile plaques, characteristic of Alzheimer's disease, are spherical, multicellular lesions containing deposits of amyloid β protein (Aβ) that include abundant amyloid fibrils intermixed with non-fibrillar forms of this peptide. These senile plaques have degenerating axons and dendrites within and intimately surrounding the amyloid deposit. Amyloid β protein is generated through the proteolytic processing of a transmembrane protein, amyloid precursor protein (APP), through β-secretase. An alternative pathway releases the amyloid sequence as a soluble amyloid protein (APPsα) through α-secretase as part of normal metabolism. APPsα administered intracerebroventrically (ICV) in mice at femtomolar doses has neurotropic effects. There is evidence that α-secretase activity is linked to muscarinic receptors and increasing muscarinic activity reduces amyloid-β deposition either as a consequence of this alone or in addition to the effects of increasing APPsα.

During natural ageing there is a general reduction in muscarinic receptor numbers in the brain. A treatment which reverses the natural decline of muscarinic receptors with age would potentially ameliorate or delay the decline in cognitive function associated with ageing as well as being another potential strategy by which to treat established Alzheimer's disease. This approach has potentially three major advantages that may modify disease progression:

- reversing the decline in muscarinic receptors to normal levels and thus increasing cholinergic activity
- increasing α–secretase activity and thereby stimulating the release of neurotrophins
- reducing amyloid-β protein formation

A screening method is known by means of which the effects of a compound under investigation on particular receptor levels may be tested. In this known method, suitable cells (typically Chinese Hamster Ovary (CHO) cells) are transfected with DNA for the receptor type of interest. The transfected CHO cells are divided into a plurality of portions for the purposes of the test and a nutrient medium is added to each portion. The cells are then allowed to grow for a predetermined time (which conventionally is determined so as to ensure that the cells do not reach confluence). The nutrient medium is then removed and replaced, in one sample, by a medium containing the test compound carried in a cytologically acceptable carrier; and, in another (control) sample, by a medium containing the carrier but not the test compound. The cell samples are then incubated for a selected time, after which the cells in the control and test samples are assessed by conventional techniques to determine the numbers of the receptor type of interest. Comparison of the results obtained from the test sample with those from the control sample then demonstrate the effect of the test compound on receptor number.

It has previously been thought to be unacceptable to allow the screening experiment to continue until the samples approach or reach a state of equilibrium; thus the conventional practice is to assess the effects of the test compound on receptor number during the active growth phase, and not when the cells are approaching or have reached equilibrium.

We believe that this standard approach is inadequate to determine whether or not a given compound may be of use in treating cognitive dysfunction, since a practical requirement for an effective treatment is to reverse the existing decline in cognitive function and any associated reduction in receptor number.

More generally, where a compound is to be tested for its efficacy in restoring depleted receptor levels, the standard approach is, we believe, defective.

Accordingly, the present invention provides a method of screening a compound to determine whether it is likely to be effective in the treatment of a condition characterised by a depletion of a specific receptor or type of receptor, which comprises the steps of:

(i) preparing or retrieving suitable cells (e.g. Chinese Hamster Ovary cells)

transfected with DNA for the specific receptor or receptor type of interest;

(ii) dividing the transfected cells into two equal portions, one portion to serve as a control sample and the other to serve as a test sample;

(iii) allowing the control sample and the test sample to grow in the presence of a nutrient medium until the cells in the samples approach or reach a state of equilibrium;

(iv) thereafter removing the nutrient medium from both of the samples and then simultaneously (a) adding to the test sample the test compound dissolved in a cytologically acceptable carrier therefor, and (b) adding to the control sample an equivalent concentration of said carrier;

(v) thereafter incubating the cells for a selected time; and

(vi) thereafter performing an assay to determine the numbers of said specific receptor or receptor type present in the cells of the control sample and of the test sample.

Typically, the cells will be allowed to grow to at least 70% confluence, and preferably at least 80% confluence or more, before the growth (nutrient) medium is removed and the subsequent steps are carried out.

The incubation period in step (v) is advantageously at least 24 hours, and may be considerably longer. Incubation times of at least 48 hours, and better still at least 72 hours, are preferred.

The cell type used in the screening method of the invention will be selected from the cell types generally used in screening and assaying techniques; CHO cells are widely used for this purpose, and the invention (although not restricted to the use of CHO cells) will be described hereinafter with reference to CHO cells as the cells of choice.

CHO cells transfected with different types of DNA may be stored at low temperatures and retrieved for subsequent use as desired.

The invention will be illustrated by the following Examples:

**Example 1**

Chinese Hamster Ovary (CHO) cells transfected with DNA for the human m2 muscarinic receptor expresses high levels of m2 receptors (~2.2 pmoles receptor/mg protein). Frozen cells (prepared previously by standard techniques) were removed from storage and plated out onto dishes or flasks at a density of approximately 15-25% confluence or higher and a conventional growth medium (containing cell nutrients including foetal calf serum and/or other appropriate nutrient factors) was added. The following day (i.e. after 24 hours) the culture medium was changed; for the test samples, a medium containing the test compound dosslved in a cytologically acceptable carrier medium (e.g. DMSO at 0.5% concentration) was added; while for the control samples, an identical medium except that it contained none of the test compound was added while,. The cells were then placed in a humidified environment and left for 48-72 hours. Medium was then removed and cell surface receptor number determined using: (i) the hydrophilic ligand, labelled N-methyl-scopolamine [$^3$H]-NMS (this measures the cell surface receptor number); and (ii) the hydrophobic ligand [$^3$H]-QNB (this measures total receptor number). Cells were washed with ice cold PBS (3x) and bound [$^3$H]-NMS determined by solubilising receptors with RIPA buffer followed by liquid scintillation counting. Non-specific binding for NMS is determined in the presence of atropine [10 μM] and for QNB by comparing with an equal number of non-transfected CHO cells.

Figure 1 of the accompanying drawings shows the results of this screening experiment when the incubation time was 1, 3 and 5 days. The CHO-m2 cells were incubated with sarasasapogenin as the test compound; this compound was selected because it is known from Chinese Patent Publication CN 1096031A to have a positive effect (an upward regulation towards normalisation) on receptor number in mammalian cells demonstrating a pathological fall in receptor number. The sarsasapogenin was present at a concentration of 10 μM. The results show that, in the control sample, prolonged culturing resulted in significant fall in the m2-muscarinic receptor number on days 3 and 5. Sarsasapogenin (10μM) incubated for up to 5 days attenuates the fall off in receptor number. Experiments were also run out to 10 days where an increase in receptor of 73±3.7 % from that of control was observed. All experimental data presented is the mean ± S.E.M. of three

determinations carried out in duplicate.

Figure 2 of the accompanying drawings shows the difference in m2 receptor number compared to the control following prolonged incubation with sarsasapogenin (10μM) in the experiment of Example 1.

**Example 2**

Incubation of cells with compounds: Cells were plated on 24 well plates 24 hours before the start of the experiment and were allowed, during this time, to grow. At the end of this time, the cells had reached at least 80% confluence. The medium was then removed and replaced with medium containing vehicle only (DMSO @ 0.5%) or medium containing the same vehicle carrying a test compound e.g. sarsasapogenin (10mM). The cells were incubated for 48 hours, then after a medium change, cells were incubated for a further 72 hours. Results analogous to those given above in Example 1 were obtained.

In a preferred embodiment, this invention provides a predictive cell based assay *in vitro* to assess putative agents for the treatment of Alzheimer's disease by prevention of and/or reversal of the decline in muscarinic receptor number. Compounds e.g. sarsasapogenin shown to be effective in the screening method of this invention are also effective in animal models of cognitive dysfunction where these compounds show a reversal of the decline in receptor number observed.

CLAIMS:

1. A method of screening a compound to determine whether it is likely to be effective in the treatment of a condition characterised by a depletion of a specific receptor or type of receptor, which method comprises the steps of:

(i) preparing or retrieving suitable cells (e.g. Chinese Hamster Ovary cells) transfected with DNA for the specific receptor or receptor type of interest;

(ii) dividing the transfected cells into two equal portions, one portion to serve as a control sample and the other to serve as a test sample;

(iii) allowing the control sample and the test sample to grow in the presence of a nutrient medium until the cells in the samples approach or reach a state of equilibrium;

(iv) thereafter removing the nutrient medium from both of the samples and then simultaneously (a) adding to the test sample the test compound dissolved in a cytologically acceptable carrier therefor, and (b) adding to the control sample an equivalent concentration of said carrier;

(v) thereafter incubating the cells for a selected time; and

(vi) thereafter performing an assay to determine the numbers of said specific receptor or receptor type present in the cells of the control sample and of the test sample.

2. A method according to claim 1, wherein said transfected cells are allowed to reach at least 70% confluence in step (iii).

3. A method according to claim 2, wherein said transfected cells are allowed to reach at least 80% confluence in step (iii).

4. A method according to claim 1, 2 or 3, wherein the incubation period in step (v) is at least 24 hours.

5. A method according to claim 4, wherein said incubation period is at least 48 hours.

6. A method according to claim 5, wherein said incubation period is at least 72 hours.

7. A screening method according to claim 1 and substantially as hereinbefore described.

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**Application No:** GB 9923075.7 **Examiner:** Dr Rowena Johnson
**Claims searched:** All **Date of search:** 2 June 2000

# Patents Act 1977
# Search Report under Section 17

**Databases searched:**

UK Patent Office collections, including GB, EP, WO & US patent specifications, in:

UK Cl (Ed.R): C3H (HB7P)

Int Cl (Ed.7): C07K 14/47, 14/705; C12N 5/06, 15/85; G01N 33/60, 33/94

Other: ONLINE: WPI, JAPIO, EPODOC, TXTE, MEDLINE, SCISEARCH, BIOSIS, EMBASE, CAPLUS

**Documents considered to be relevant:**

| Category | Identity of document and relevant passage | Relevant to claims |
|---|---|---|
| X | *Life Sci;* Vol 62 (5), pp 389-396 (1998). Fukamauchi *et al.* Entire document, but see especially materials and methods *cell culture* and results page 391. | 1-7 |
| X | *Biochem. Pharmacol.;* Vol 54 (7), pp 833-839 (1997). Chang *et al.* Entire document, but see especially page 834, 2nd paragraph, and discussion. | 1-7 |
| X | *Mol. Pharmacol.;* Vol 44 (5), pp 940-949 (1993). Fukamauchi *et al.* Entire document, but see especially results. | 1-7 |
| X | *Febs Letts;* Vol 276 (1-2), pp 185-188 (1990). Wang *et al.* Entire document, but see especially results and discussion . | 1-7 |

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